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Enhancing Tumor Targeting and Apoptosis Using Noncovalent Antibody Homodimers

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Summary: A rare type of antibody that spontaneously binds to each self (homodimerizes) has been described. This self-binding (autophilic) antibody provides stronger protection against bacterial infection than a non self-binding antibody with identical specificity and affinity due to increase of polymeric avidity. A peptide derived from the self-binding domain of the autophilic antibody was crosslinked to the Fc carbohydrate of two monoclonal antibodies specific for the B-cell receptor (BCR) of a murine and a human B-cell tumor. Peptide-crosslinked antibodies bind to themselves on solid phase ELISA as homodimer and establish in solution a monomer-dimer equilibrium. Autophilic antibodies bind to their respective tumor target cells with increased efficiency as determined by FACS analysis. They also induce twice the amount of apoptosis of target tumor cells than the control antibodies. Furthermore, the modified antibodies inhibit tumor growth in culture more efficiently than the control antibodies. Criss-cross protocols in FACS, apoptosis, and growth inhibition indicate the specificity of targeting the BCR with autophilic antitumor antibodies. The chemical approach of increasing the binding of antibodies without creating chemically crosslinked dimers mimics naturally occurring autophilic antibodies and represents a simple and attractive alternative to chemical dimerizing and antibody engineering techniques for improving their antitumor effect. Furthermore, these results provide a guide to incorporate the self-binding peptide into the structure of antibodies using modeling and molecular crafting techniques. **Key Words:** Antibody—Apoptosis—B-cell lymphoma—Homodimer.

the variable domain of the heavy chain and comprises regions of CDR2 and FR3 of the germline encoded S107/TEPC15 (T15) antibody. The autophilic site is present on antibodies with different specificity (5) rendering its expression independent of antigen driven selection. Because of the antigen-independent expression of the autophilic effect, we considered the possibility to attach the autophilic peptide to monomeric antibodies and to create polymeric antibodies with increased avidity. This hypothesis was tested by affinity crosslinking the autophilic T15 peptide to antibodies against the B-cell receptor (BCR) of lymphoma tumors.

The antitumor effect of antibodies against the BCR is mediated by negative signaling requiring crosslinking of the BCR (6–9). This signal for growth arrest and apoptosis (10,11) of the B-cell tumor is greatly enhanced by homodimerizing antibodies that occur either spontaneously (12) or can be manufactured by chemical crosslinking (13).

In a recent study (14), we have generated an autophilic antibody against CD-20 of human B-cell lines and shown enhanced antitumor effects. Here we extended this approach using antibodies against the BCR molecule of a human and a murine B-cell line and demonstrate a monomer-dimer equilibrium of autophilic antibodies in solution that are not chemically crosslinked.

MATERIALS AND METHODS

Cell Line and Antibodies

The human B-cell tumor line (Su-DHL4) and murine B-cell tumor line (38C13) are grown in RPMI 1640 medium (supplemented with 10% fetal bovine serum, 2 μmol/L glutamine, 10 μmol/L HEPES, 50 U/mL penicillin, and 50 μg/mL streptomycin, 50 μmol/L 2-mercaptoethanol) at 37°C under 5% CO_2. Two mAb 5D10 and S1C5, specific for the human or murine BCR, respectively, were used in this study. The antibodies are purified from the culture supernatant by protein G and protein A affinity chromatography.

Synthesis of Antibody-Peptide Conjugate

T15H peptide (ASRNKANDYTTDYSASVKGRFIVSR), a V_H-derived peptide from a self-binding antibody-T15 (5), was synthesized by Genemed Synthesis (San Francisco, CA, U.S.A.). Antibodies were dialyzed against PBS (pH6.0) and 1/10 volume of 200 μmol/L $NaIO_4$ was added and incubated at 4°C for 30 minutes in the dark. The reaction was stopped by adding glycerol to 30 μmol/L, and the sample was dialyzed at 4°C for 30 minutes against PBS (pH 7.0). 100 times molecular excess of T15H or scrambled peptide was added to the antibodies and incubated at 37°C for 1 hour. L-Lysine was added and incubated at 37°C for 30 minutes to block the remained aldehyde group. The same oxidation reaction steps (except adding the peptides) were applied to antibodies used as controls. After the blocking step, the antibody-conjugates were dialyzed against PBS (pH 7.2) overnight.

Ig Capture ELISA

Four μg/mL of S1C5-T15H was coated to Costar vinyl assay plates (Costar, Cambridge, MA, U.S.A.). After blocking with 3% BSA solution, 8 μg/mL of photobiotinylated S1C5-T15H (15), S1C5-scrambled peptide conjugate, and control S1C5 were added to the first wells, and 1:1 dilution was performed. The antibodies were incubated for 2 hours at room temperature. After washing with PBS buffer, Avidin-HRP (Sigma, St. Louis, MO, U.S.A.) was added as a 1:2500 dilution. The binding antibodies were visualized by adding substrate *o*-phenylenediamine.

Size Exclusion Chromatography

Antibody conjugate was chromatographed on a 75 mL Sephacryl 300HR column (Pharmacia, Peapack, NJ, U.S.A.). 1:10 diluted PBS (pH 7.2) was chosen as elution buffer. Fractions (0.5 mL/each) were collected and aliquots (100 μL) were assayed on antihuman IgG capture ELISA. The ELISA reading (OD 490 nm) is plotted against elution volume.

Viability Assay for Antibody-Treated Cells

The lymphoma cells were grown in 96-well tissue culture wells in 1-mL medium. 2 μg of antibodies or antibody-peptide conjugates were added and incubated for various times as described in the "RESULTS" section. Ten μL aliquots from the cell suspension were used to determine viability by using trypan blue exclusion.

FACS Assay of the B-Cell Lymphoma

The Su-DHL4 and 38C13 cells were fixed with 1% paraformaldehyde. 1×10^6 cells were suspended in 50 μL of staining buffer (Hank's balanced salt solution, containing 0.1% NaN_3, 1.0% BSA), then 1.5 μg of photobiotinylated S1C5-T15H conjugates (15) was added and incubated for 30 minutes on ice. Control antibodies and antibody-scrambled T15 peptide conjugates served

as controls. The cells were washed twice with staining buffer before Avidin-FITC (Sigma) was added to the cells for 30 minutes on ice. Then the cells were washed twice with staining buffer, resuspended in 200 μL PBS and analyzed by flow cytometry.

Hoechst-Merocyanin 540 Staining to Detect Apoptosis

1×10^6 of lymphoma cells were placed into 24-well tissue culture wells. Four μg of antibodies or antibody-peptide conjugates were added and incubated for various times as described in the "RESULTS" section. 1×10^6 cells were removed from the culture, resuspended in 900 μL cold PBS (pH 7.2). One hundred μL of Hoechst 33342 (50 μg/mL; Molecular Probe, Eugene, OR, U.S.A.) was added, the cells were incubated at 37°C for 30 minutes in the dark. The cells were centrifuged and resuspended in 100 μL PBS. Then, 4 μL of MC540 solution (Molecular Probe) was added, and a 20-minute incubation was performed at room temperature in the dark. The cells were pelleted, resuspended in 1 mL cold PBS (pH 7.2), and analyzed by flow cytometry (16).

RESULTS

Characterization of Autophilic Antibodies

The T15H (24-mer) peptide (4) was crosslinked to two murine mAb (S1C5 and 5D10), using carbohydrate periodate conjugation (17). The mAb S1C5 (IgG1) is specific for the tumor idiotype of the mouse 38C13 B-cell line (18) and the 5D10 antibody for the human Su-DHL4 B-cell tumor (19). Both antibodies recognize unique idiotypes of the BCR IgM on the B-cell tumors.

Self-Binding Behavior can Easily be Demonstrated by ELISA

The autophilic self-binding effect was studied with the T15H peptide-crosslinked mAb S1C15. As seen in Figure 1A (panel A), the T15H-crosslinked S1C5 binds to insolubilized S1C5-T15H detected by biotin-avidin ELISA. Control S1C5 does not bind significantly to S1C5-T15H or S1C5 crosslinked with a scrambled peptide. Similar self-binding of T15H peptide-crosslinked mAb 5D10 to insolubilized T15H-5D10 was also observed (Fig. 1A [panel B]).

The specificity of the peptide mediated autophilic effect was tested using the 24 mer peptide T15H itself as an inhibitor. As seen in Figure 1B, only the T15H peptide inhibited S1C5-T15H (Fig. 1B [panel A]) and 5D10-T15H (Fig. 1B [panel B]) self-binding while the control scrambled peptide did not inhibit it. These results are similar to the previously published inhibition data with the naturally occurring autophilic T15/S107 antibody (4,5).

T15H-Antibody Conjugates Form an Equilibrium of Monomer and Dimer in Solution

The noncovalent nature of the self-aggregation of T15H-linked antibodies raises the question of its physical state in solution. To address this issue, we analyzed the molecular species of T15H-linked mAb using gel electrophoresis and sizing gel filtration. In Figure 1C, the electrophoretic mobility of control and T15H peptide-conjugated S1C5 and 5D10 under reducing and nonreducing conditions show no differences, indicating the absence of chemical bonds between the antibody chains. The faint band in lanes 3 and 4 of Figure 1C represent dimers of T15H-linked mAb.

The molecular species of the peptide conjugated antibodies (5D10-T15H) was further analyzed by size exclusion chromatography. In Figure 2, representative results are shown. The elution profile indicated two immunoglobulin species of different size (Fig. 2A). The second smaller peak eluted in the position of nonconjugated 5D10 antibody. The larger first peak eluted in the position of an antibody dimer. The appearance of two peaks resembled monomer and dimer antibodies and could indicate that either a fraction of antibodies was not modified to polymerize, or that the modification was complete and the antibody establishes an equilibrium of dimers and monomers. To test the latter possibility, material from both peaks were subjected to a second gel filtration on the same column. Reruns of both peaks yielded again two peaks (Figs. 2B and C) at the same position as in the first chromatography. The inserts in Figure 2 show the SDS-PAGE of the material from peak I and II, indicating the typical heavy (H) and light (L) chain bands of antibodies. These data show that the T15H peptide-linked antibodies exist in solution as two distinct molecular species that form an equilibrium of monomer and dimer. This behavior is reminiscent of the gel filtration data on the natural self-binding antibody T15 (20).

Enhanced Binding of Autophilic Antibodies to Tumors

The binding of the peptide conjugated antibodies against their respective tumor targets was compared with that of the control antibodies in indirect fluorescence activated cell sorting (FACS). As control, antibodies

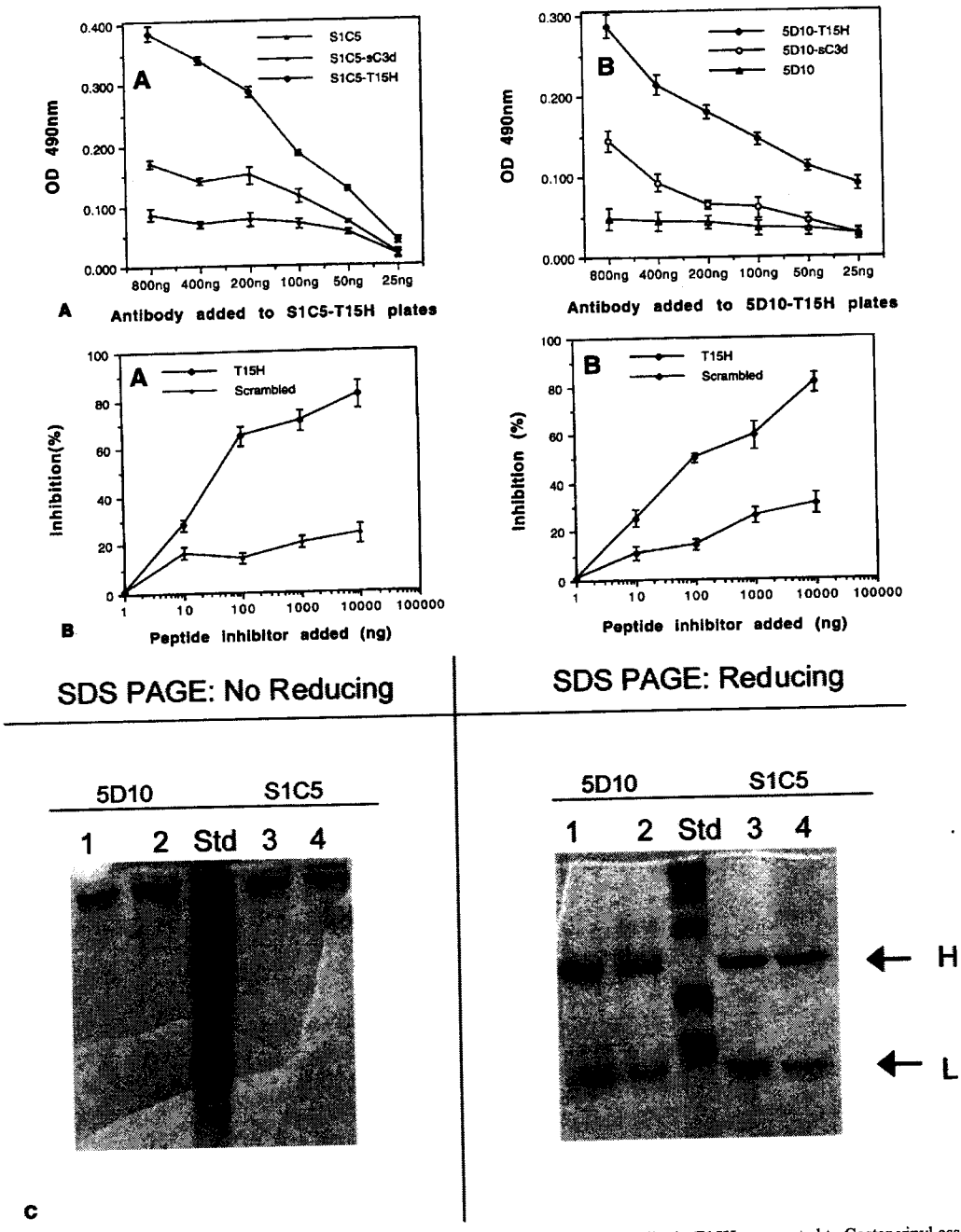

FIG. 1. **(A)** Self-binding of autophilic S1C5 (panel A) and 5D10 (panel B). Four μg/mL of antibody-T15H was coated to Costar vinyl assay plates. After blocking with 3% BSA solution, 8 μg/mL of photobiotinylated antibody-T15H, antibody-scrambled peptide conjugate, and control antibody were added to the first wells, and 1:1 dilution was performed. After a 2-hour incubation at room temperature, Avidin-HRP was added as a 1:2500 dilution. The binding antibodies were visualized by adding substrate *o*-phenylenediamine. **(B)** Inhibition of self-binding. Four μg/mL of photobiotinylated antibody-T15H was mixed with different concentrations of T15H or scrambled peptide and added to antibody-T15H coated plates (400 ng/well), incubated for 2 hours at room temperature, and the binding was developed as described in **(A)**. **(C)** SDS-PAGE of autophilic antibodies. PAGE in reducing and nonreducing conditions of nonmodified and peptide-conjugated mAb S1C5 and 5D10. Lanes 1 and 3 show the control antibodies, lanes 2 and 4 are the peptide-conjugated antibodies; Std, molecular weight standard marker.

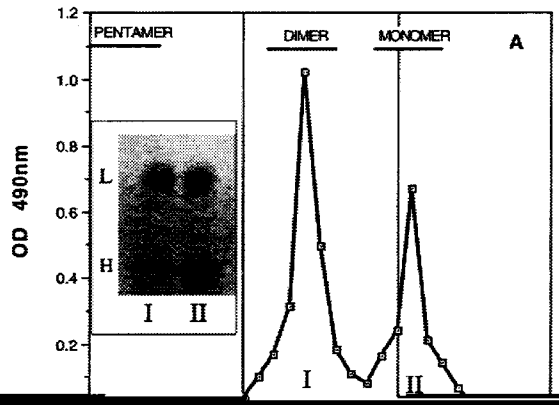

FIG. 2. Size exclusion chromatography of 5D10-T15H. Antibody conjugates were chromatographed on a 75-mL Sephacryl 300HR column. 1:10 diluted PBS (pH 7.2) was chosen as elution buffer. Fractions (0.5 mL/each) were collected and aliquots (100 μL) were assayed on antihuman IgG capture ELISA. The ELISA reading (OD 490nm) is plotted against elution volume. The elution of IgM, dimer IgA, and monomer IgG are indicated in **(A)**. Inserts show the SDS-PAGE of the materials from the peak fractions I and II; H and L indicate the heavy and light chain bands. **(A)** Elution profile of 5D10-T15H. **(B)** Elution profile of peak I. **(C)** Elution profile of peak II.

linked with a scrambled peptide were included. In Figure 3A, the fluorescence intensity of the T15H-S1C5 on 38C13 cells (Fig. 3A [panel D]) is compared with that by

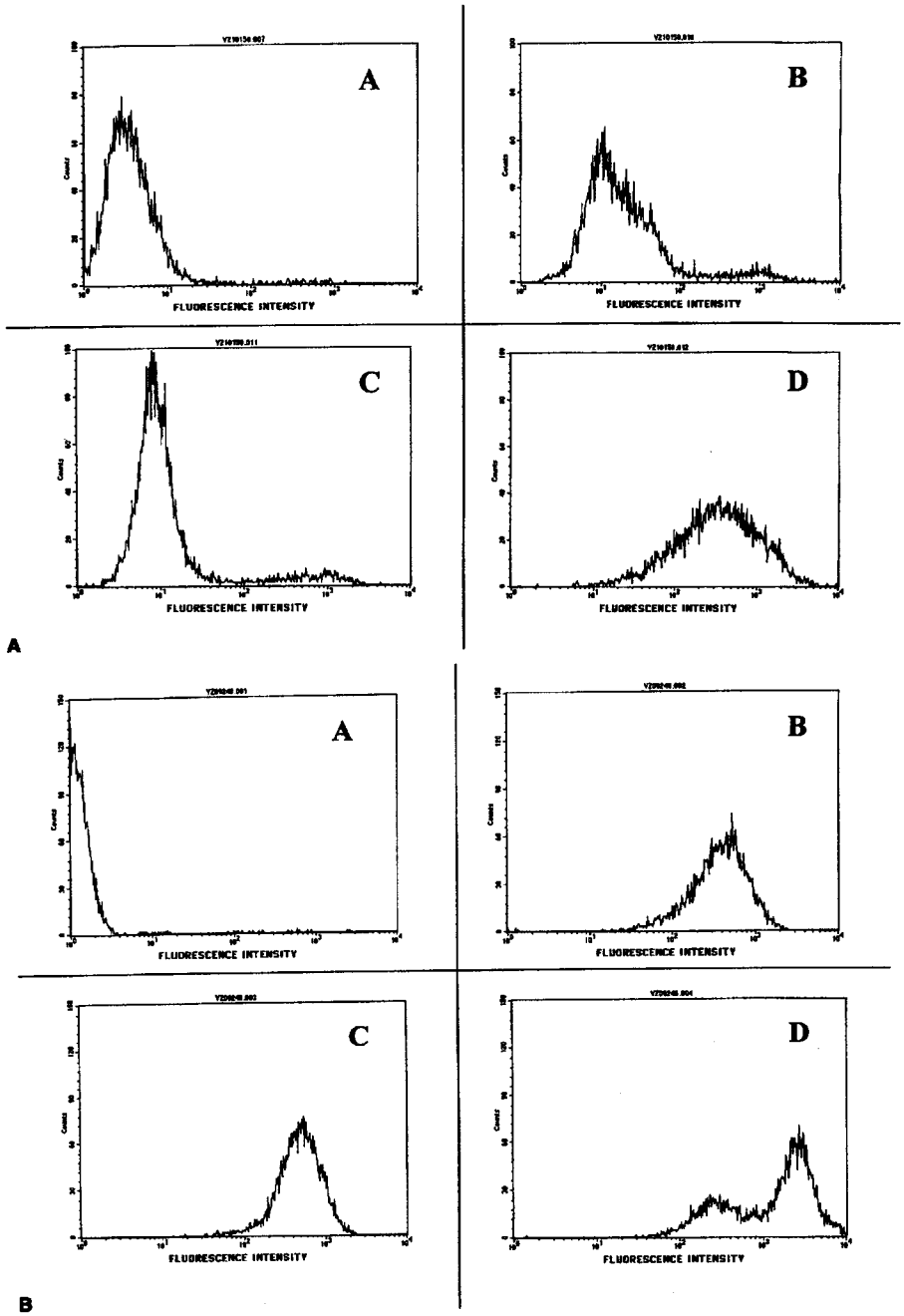

FIG. 3. FACS analysis of tumor cells after incubation with modified antibodies. 1×10^6 cells were suspended in 50 μL of staining buffer, then 1.5 μg of photobiotinylated S1C5-T15H conjugates was added and incubated for 30 minutes on ice. Control antibodies and antibody-scrambled T15 peptide conjugates served as controls. After washing step, avidin-FITC was added to the cells for 30 minutes on ice. After washing, the cells were resuspended in 200 μL PBS and analyzed by flow cytometry. **(A)** 38C13 cells. (Panel A) No primary antibody; (panel B) stained with S1C5; (panel C) Stained with S1C5-scrambled peptide conjugate; (panel D) stained with S1C5-T15H peptide conjugate. **(B)** The Su-DHL4 cells. (Panel A) No primary antibody; (panel B) stained with 5D10; (panel C) stained with 5D10-scrambled peptide conjugate; (panel D) stained with 5D10-T15H conjugate.

[panel A]). Similar results on the specificity of autophilic antibodies were obtained with the Su-DHL4 cells (Fig. 4B [panel B]).

Induction of Apoptosis

As suggested by earlier studies (21–23), the antitumor effect of antibodies directed against the BCR of B-cell lymphomas in vitro and in vivo might be caused by the induction of apoptosis. Aliquots of tumor cells (38C13 and Su-DHL-4) cultured in the presence of control or T15H-linked antibodies were analyzed for apoptosis using a double stain FACS protocol (16). The results are T1 summarized in Table 1. 38C13 and Su-DHL4 cells underwent a moderate amount of apoptosis without antibodies over a 6, respectively 18-hour culture. This apoptosis was enhanced when the respective antibody was added. However, when the T15H-linked antibodies were added, the accumulated number of apoptotic 38C13 cells was almost doubled, and apoptosis of Su-DHL4 cells was more than doubled during the entire culture.

ED1 **TABLE 1.** *Analysis of apoptotic cells*

Tumor cells*	38C13		Su-DHL4	
Culture time (hours)	3	6	9	18
Antibodies	None		None	
R1: G_0/G_1 viable	58.24†	61.21	57.75	44.29
R2: $S/G_2/M$ viable	34.05	30.07	33.68	49.38
R3: G_0/G_1 apoptotic	2.97	3.40	3.91	1.16
R4: S/G2/M apoptotic	2.63	3.31	3.52	1.55
R5: Fragmented	0.57	0.97	0.08	0.24
Σ(R3,R4,R5): apoptotic	6.17	7.68	7.51	2.95
Antibody	S1C5		5D10	
R1: G_0/G_1 viable	61.87	57.29	57.14	44.41
R2: $S/G_2/M$ viable	27.68	27.29	31.84	43.58
R3: G_0/G_1 apoptotic	3.99	4.59	5.40	1.93
R4: S/G2/M apoptotic	2.80	4.71	4.83	1.87
R5: Fragmented	2.91	5.56	0.18	1.43
Σ(R3,R4,R5): apoptotic	9.7	14.86	10.41	5.23
Antibody-T15H	T15-S1C5		T15-5D10	
R1: G_0/G_1 viable	49.72	45.18	48.98	44.68
R2: $S/G_2/M$ viable	30.21	23.02	30.55	26.17
R3: G_0/G_1 apoptotic	7.42	7.18	7.75	10.90
R4: S/G2/M apoptotic	7.45	10.47	6.82	9.01
R5: Fragmented	5.80	6.85	1.88	1.76
Σ(R3,R4,R5): apoptotic	20.67	24.5	16.45	21.67

* 38C13 and Su-DHL4 cells were cultured in absence and presence of 2 μg of antibody or antibody peptide conjugate. After a certain time (3 and 6 hours for 38C13; 9 and 18 hours for SU-DHL4) of incubation, the cells were stained with Hochest 342 and MC540 and analyzed by flow cytometry as described (15).

† Percent of analyzed cells (10,000): R1, cells with 2n DNA that are MC 540 negative; R2, cells with greater than 2n DNA that are MC 540 negative, R3, cells with 2n DNA that are MC 540 bright; R4, cells with greater than 2n DNA that are MC 540 bright; R5, cells displayed

DISCUSSION

The biologic advantage of the autophilic property is exemplified with the S107/T15 antiphosphorylcholine antibody (2). This self-binding antibody is several times more potent in protecting immune-deficient mice against infection with pneumococci pneumoniae (24,25) than nonself-binding antibodies with the same antigen specificity and affinity.

As shown here, the autophilic antibody function can be transferred to other antibodies by chemically crosslinking a peptide derived from the T15 V_H germline sequence. The modified antibody mimics the self-binding property of the T15/S107 antibody (2), producing a dimeric antibody with increased avidity and enhanced targeting. This approach is an attractive alternative to strategies of improving the targeting of antibodies by either chemical crosslinking or by antibody engineering.

Enhancing the binding of autophilic engineered antibodies to the BCR of B-cell tumor increases the strength of the death signals leading to profound inhibition of cell proliferation in culture. Even though the doubling of apoptosis is demonstrated here, it appears that other mechanisms of growth inhibition are involved.

Crosslinking the BCR of the mature murine B-cell lymphoma A20 can protect against CD95 mediated apoptosis (26). This antiapoptotic activity of engagement of the BCR by crosslinking antibodies is highly restricted to the time window of CD95 stimulation and is not dependent upon protein synthesis (27). Our finding that BCR hypercrosslinking per se is pro-apoptotic is not at variance with reports on the antiapoptotic activity of the BCR engagement, because it can be a result of the use of less mature B-cell lines in our study, to different strength of delivered signals by homodimerizing antibodies, or to Fas independent apoptosis (23,28,29).

The use of two BCR idiotope specific antibodies against different tumors offered the opportunity to test the biologic effect of targeting receptors other then the idiotope specific BCR. In criss-cross experiments with autophilic antibodies binding in FACS analysis and inhibition of growth in vitro show a significant enhancement only with the autophilic matched antibody. In this context, it is interesting to speculate whether enhanced tumor targeting would also augment cellular effector functions. Such in vitro and in vivo experiments are in progress.

In an earlier study (13) using chemically homodimer-

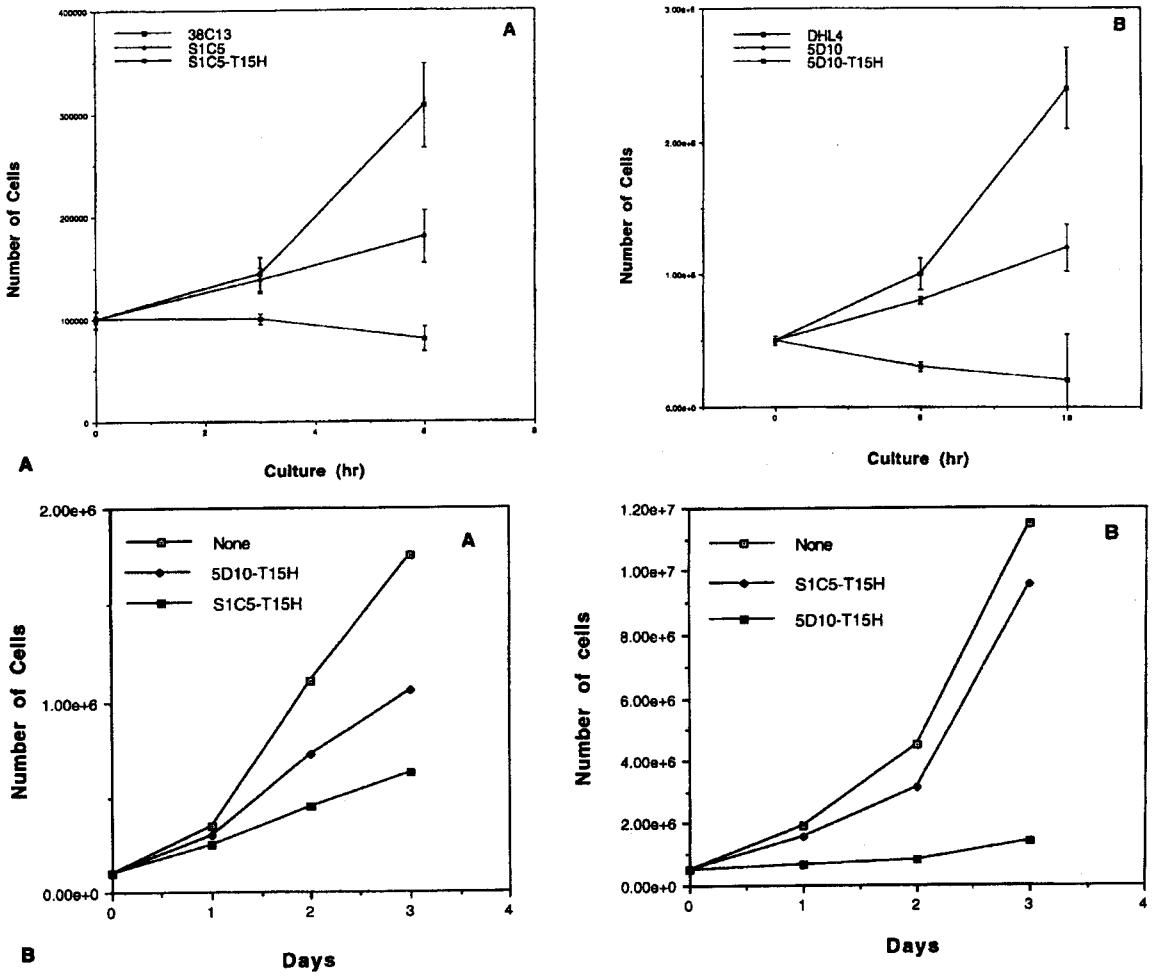

FIG. 4. Inhibition of tumor growth. **(A)** 38C13 (1×10^5/mL) (panel A) and SU-DHL4 cells (5×10^5/mL) (panel B) were cultured in 1 mL tissue culture wells; 2 μg of antibodies or (autophilic) T15H-antibody were added. After 3 and 6 hours (for 38C13) and 9 and 18 hours (for Su-DHL4) of incubation, the cell viability was determined by trypan blue exclusion, The number of viable cells is plotted at different hours of culture. **(B)** Criss-cross tumor growth inhibition. 38C13 (1×10^5/mL) (panel A) and SU-DHL4 cells (5×10^5/mL) (panel B) were cultured in presence of S1C5-T15H and 5D10-T15H antibodies as in **(A)**. *No statistically significant differences between the unmatched antibody and no antibody control, $p \geq 0.1$.

ized antibodies, the Fc domain was not involved in the augmentation of growth inhibition and tumor cells lacking Fc receptors were susceptible to the antigrowth activity of homodimers. Thus, the antitumor effect induced by dimerizing antibodies would not be restricted to tumors expressing Fc-receptors (30,31).

The described approach of transferring the naturally occurring autophilic property to other antibodies thereby enhancing their antitumor effect outlines a general method to improve the therapeutic efficacy of antibodies in passive immunotherapy. Such noncovalent antibody complexes offer several advantages over chemically crosslinked antibodies: (i) the equilibrium between monomer and noncovalent homopolymers prevents the formation of precipitating nonphysiologic complexes in solution (this report and [20]); (ii) autophilic conversion does not compromise the structural integrity of antibodies; and (iii) the method is simple and efficient and does not require a purification step typically needed for chemically crosslinked homodimers that reduces the yield of active Ig dimers. One possible limitation of the approach of using dimerizing antibodies might be the ability to penetrate a large tumor mass. Because the homophilic peptide is of murine origin, it might be immu-

nogenic in humans. Thus, it could be necessary to humanize the murine peptide based on sequence and structural homology using computer modeling. The demonstration that adding a single peptide to the structure of antibodies increases the amount of antibody bound to targets and the antitumor activity encourages attempts to engineer recombinant antibodies expressing

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